

## Effector-Mediated Eradication of Precursor B Acute Lymphoblastic Leukemia with a Novel Fc-Engineered Monoclonal Antibody Targeting the BAFF-R

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### Abstract

B-cell activating factor receptor (BAFF-R) is expressed on precursor B acute lymphoblastic leukemia (pre-B ALL) cells, but not on their pre-B normal counterparts. Thus, selective killing of ALL cells is possible by targeting this receptor. Here, we have further examined therapeutic targeting of pre-B ALL based on the presence of the BAFF-R. Mouse pre-B ALL cells lacking BAFF-R function had comparable viability and proliferation to wild-type cells, but were more sensitive to drug treatment *in vitro*. Viability of human pre-B ALL cells was further reduced when antibodies to the BAFF-R were combined with other drugs, even in the presence of stromal protection. This indicates that inhibition of BAFF-R function reduces fitness of stressed pre-B ALL cells. We tested a novel humanized anti-BAFF-R monoclonal antibody optimized for FcγRIII-mediated, antibody-dependent cell killing by effector cells. Antibody binding to human ALL cells was inhibitable, in a dose-dependent manner, by recombinant human BAFF. There was no evidence for internalization of the antibodies. The antibodies significantly stimulated natural killer cell-mediated killing of different human patient-derived ALL cells. Moreover, incubation of such ALL cells with these antibodies stimulated phagocytosis by macrophages. When this was tested in an immunodeficient transplant model, mice that were treated with the antibody had a significantly decreased leukemia burden in bone marrow and spleen. In view of the restricted expression of the BAFF-R on normal cells and the multiple anti-pre-B ALL activities stimulated by this antibody, a further examination of its use for treatment of pre-B ALL is warranted. *Mol Cancer Ther*; 1–11. ©2014 AACR.

### Introduction

Although the overall cure rate for adult precursor B-lineage acute lymphoblastic leukemia (pre-B ALL) has increased from 43% to 57% in the period between 1987 and 2007, a need remains for the development of more effective and less toxic treatments. The identification of targets for therapy that are expressed on a limited subset of

cells, thus minimizing therapy side effects, is an important component of this. We were the first to report that pre-B ALL cells aberrantly express the B-cell activating factor receptor (BAFF-R; ref. 1). This is the principal receptor for BAFF, a TNF family member and a type II transmembrane protein found either in a membrane bound or soluble form on dendritic cells, stromal cells, macrophages, and some T cells (2). The receptor for BAFF is expressed on normal immature and mature B cells, as well as on other malignant B-lineage cells, including chronic lymphocytic leukemia, myeloma, hairy cell leukemia, and B lymphoma (3–5). The BAFF-BAFF-R interaction is critical for survival of mature B cells, because profound loss of these cells is observed in both *baff* and *baff-r* null mutant mice. In normal mature B cells, the interaction between the ligand BAFF and the BAFF-R enhances B-cell survival (6). Also, intracellular signal transduction cross-talk occurs between the B-cell receptor (BCR) and the BAFF-R, and BCR stimulation induces *BAFF-R* mRNA expression in mature B cells (7, 8).

Although the functional significance of the presence of the BAFF-R on pre-B ALL cells was unclear, the finding that normal pre-B cells lack BAFF-R expression (1, 9, 10) makes this receptor an attractive target for ALL therapy. In one such approach, using a recombinant fusion protein

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between BAFF and the toxin Gelonin, we recently demonstrated that the presence of the BAFF-R can be used to selectively eradicate pre-B ALL cells (11).

Other potential therapeutic approaches showing promise include using monoclonal antibodies (mAbs) as immunotoxin conjugates or bispecific antibodies (5, 12–14). A different mechanism by which antibodies can be used to target cancer cells for eradication is through antibody-dependent cellular cytotoxicity (ADCC). This is mediated mainly by Fc $\gamma$ RIII, a major triggering receptor on natural killer (NK) cells. Several therapeutic mAbs in use for cancer treatment mediate ADCC, including anti-CD20 rituximab (Rituxan), anti-Her2 trastuzumab (Herceptin), anti-TNF- $\alpha$  infliximab (Remicade), and anti-RhD (15). ADCC-promoting antibodies that were developed for more mature B-cell cancers such as rituximab, alemtuzumab, and epratuzumab are also being tested for treatment of B-cell precursor ALL (16, 17). Antibody coating of cells can also stimulate antibody-dependent cell-mediated phagocytosis (ADCP) by macrophage effector cells. Interestingly, two preclinical studies reported different outcomes using mAbs to stimulate effector-mediated eradication of precursor B-lineage ALL cells. Three mixed-lineage leukemia (MLL)-positive ALL cell lines were resistant to NK-mediated ADCC in the presence of a CD19 antibody (13). The second study reported that Medi-551, a humanized anti-CD19 mAb, stimulates both ADCC by NK cells and phagocytosis by macrophages (18).

Although many antibodies generated against the BAFF-R inhibit BAFF-mediated B-cell growth *in vitro* (19), to date, none of these were reported to be successful in therapeutical applications. In the current study, using a novel BAFF-R antibody, optimized for ADCC, we demonstrate that this receptor is an extremely attractive target for effector-mediated eradication of pre-B ALL cells.

## Materials and Methods

### Reagents

Nilotinib and anti-BAFF-R B-1239 antibodies (unconjugated and Alexa-647 labeled) were from Novartis. These antibodies were selected from the Human Combinatorial Antibody Library (HuCAL GOLD(r)), using phage display technology (8). The B-1239 mAb was produced in a fucosyl-transferase-deficient CHO cell line (BioWa Potelligent Technology; BioWa Inc.; ref. 20), with a humanized sequence to optimize ADCC activity. B-1239 was selected based on its property of blocking the BAFF/BAFF-R interaction and signaling via BAFF. Anti-BAFF-R antibody clone 11c1, anti-human CD19, and CD10 antibodies were from BD Biosciences. Fluorescein isothiocyanate (FITC) anti-human immunoglobulin G (IgG) was from Sigma Aldrich. Recombinant huBAFF and the function-blocking anti-BAFF-R antibodies were purchased from R&D Systems. Bcr N-20, BAFF-R, and Gapdh antibodies for Western blotting

were from Santa Cruz, eBiosciences, and Millipore, respectively. Cell Mask Orange and Deep Red were from Life Technologies.

### Mouse pre-B ALL wild-type and KO cells, human patient-derived and primary samples

*baff-r*-deficient and control C57Bl/6J mice were purchased from The Jackson Laboratory. Bcr/Abl-IRES-neo (p190), pHIT60, and pHIT123 (ecotropic envelope) plasmids were transfected to HEK 293FT cells using Lipofectamine 2000 (Life Technologies). Viral supernatant collected after 24 hours was transferred to a 6-well plate coated with retronectin (Takara). After centrifuging the viral supernatant at 2,000 g for 90 minutes, pre-B cells were added onto the plates and again centrifuged at 600 g for 30 minutes. Total bone marrow (BM) cells were cultured in the presence of 10 ng/mL recombinant mouse interleukin (IL)-7 for 12 days, after which cells were retrovirally transduced and IL-7 was withdrawn. We transformed bone marrows of three independent sets of wild-type (WT) and null mutant (KO) mice. US7 and US7R are Ph-negative ALLs; USFO2, BLQ1, BLQ11, P2 (Pt2, LAX2), and TXL2 are Ph-positive ALLs. BLQ1, BLQ11, and P2 contain a Bcr/Abl T315I mutation. ICN13 is a pro-B ALL with an MLL-AF4 fusion (1, 21, 22). TXL2R cells were derived from TXL2 and are able to proliferate in the presence of 500 nmol/L nilotinib. Human ALL patient-derived cells have been passaged in NOD/SCID/IL2 rg<sup>-/-</sup> (NSG) mice and were then grown on irradiated OP9 feeder layers. The acute myelogenous leukemia (AML) was from a relapsed patient and grew directly in culture when provided with OP9 stromal support. Non-cultured primary human ALL samples were obtained after approval of the Committee on Clinical Investigations of Children's Hospital Los Angeles. B-precursor ALLs [BM, peripheral blood (PB)] were gated to identify blasts and normal residual B lymphocytes (mainly mature B cells) expressing high levels of CD45. Each group was independently assessed for BAFF-R expression and mean fluorescent intensity (MFI).

### Assay for receptor density and internalization

Cells were washed with autoMACs rinsing solution containing bovine serum albumin and resuspended in human or mouse Fc $\gamma$ R blocking reagent (Miltenyi Biotech). After 15 minutes, cells were stained with 2.5  $\mu$ g/mL Alexa-647 B-1329 or hIgG1 for 30 minutes, washed, then incubated with live/dead cell stain (Invitrogen). Cells were analyzed on a Fortessa (BD). Cell surface receptor numbers were quantified by running Quantum Simply Cellular anti-IgG Beads and APC-MESF beads (Bangs Laboratories) in parallel with the cell samples.

### ImageStream and confocal microscopy analysis

US7 cells ( $3 \times 10^6$ ) were incubated for 4 hours with antibodies, washed 1 $\times$  with PBS, and then incubated for 5 minutes with plasma membrane stain. The cell surface

was defined using 0.25 µg/mL Cell Mask Deep Red with 15 µL of internalizing probe [phycoerythrin (PE)-BAFF antibodies; eBiosciences] or Cell Mask Orange with 0.6 µg/mL B-1239 Alexa 647. Acquisition was performed using an ImageStream Imaging Flow Cytometer (Amnis Corporation) using INSPIRE software. A 40× magnification was used for all samples. Data analysis was performed using the IDEAS v.6 software (Amnis Corporation). The internalization score is defined as the ratio of signal intensity inside the cell to the intensity of the entire cell for a specific fluorescent signal, with higher internalization scores indicating higher internalization.

For confocal microscopy, cells were imaged on an LSM 710 confocal system mounted on an AxioObserver.Z1 microscope equipped with a 40×/1.2 C-APOCHROMAT water-immersion lens (Carl Zeiss Microimaging). Fluorescence excitation was achieved with laser lines of 633 nm and emission ranges were 662 to 721 nm for Alexa Fluor 647. The confocal pinhole was set to 1 Airy unit. Brightfield images were acquired simultaneously with fluorescence. The confocal system was controlled by the Zeiss ZEN 2010 software. Incubation times and antibody concentrations were the same as for ImageStream analysis.

#### ***In vitro* antibody treatment**

For assessment of BAFF-R antibody binding, ALL cells were incubated with different concentrations of the B-1239 BAFF-R antibody for 30 minutes at room temperature (RT), washed with PBS and incubated with FITC-conjugated anti-human IgG antibody, and analyzed by fluorescence-activated cell sorting (FACS; Accuri Flow Cytometers Inc.). Staining of these cells with FITC anti-human IgG alone served as control. For competition assays, US7 ALL cells were preincubated with recombinant human BAFF or PE-labeled α-BAFF-R antibody (BD Biosciences) for 2 hours, followed by incubation for 30 minutes with 5 µg/mL B-1239 α-BAFF-R antibody. In combination treatments, antibody and drug were added every alternate day. Non-adherent leukemia cells were collected from the stromal layer and viability of ALL cells was determined by manually counting Trypan blue-excluding lymphoblasts under a microscope.

#### **ADCC assays**

NK cells from normal human peripheral blood were isolated using a MACS negative separation column (Miltenyi Biotech). ALL cells ( $10^6$ ) were labeled with calcein-AM (Lifescience Technologies) for 30 minutes at 37°C. Control human IgG Ab or B-1239 α-BAFF-R-Ab was added at 20,000, 10,000, 1,000, 100, and 10 ng/mL to the ALL cells. After 1 to 2 hours of incubation, cells were washed with PBS<sup>-/-</sup> and counted. A total of 10,000 cells per well were added to a 96-well plate. MACS-sorted human NK cells isolated from peripheral blood were added at 50,000/well [effector-to-target

(E:T) ratio = 5:1]. The optimal E:T ratio of 5:1 was determined in pilot experiments. After 4 hours of incubation, 100 µL of culture supernatant was transferred to a Black View Plate-96 well plate and arbitrary fluorescent units (AFU) were measured on a Tecan SPECTRA-FLUOR (485-nm excitation/535-nm emission). The percentage of specific lysis from triplicate wells was determined using the following equation, in which "AFU mean spontaneous release" is calcein-AM release by target cells in the absence of antibody or NK cells and "AFU mean maximal release" is calcein-AM release by target cells upon lysis by detergent.

$$\% \text{ specific lysis} = 100 \times (\text{AFU mean experimental release} - \text{AFU mean spontaneous release}) /$$

$$(\text{AFU mean maximal release} - \text{AFU mean spontaneous release})$$

#### **ADCP assays**

Human mononuclear cells were isolated from normal PB by Ficoll centrifugation. Cells were plated on non-tissue culture-treated 10-cm dishes and activated with 100 nmol/L phorbol 12-myristate 13-acetate for 1 hour. Floating cells were washed off and remaining adherent cells propagated for at least 6 days with fresh media (RPMI, 15% FBS, penicillin/streptomycin, β-mercaptoethanol) added every other day. For measurement of ALL eradication by human macrophages, leukemia cells were first incubated for 30 minutes with 25 µg/mL B-1239. A total of  $5 \times 10^5$  washed ALL cells were then plated into wells containing  $10^5$  macrophages. Remaining Trypan blue excluding live cells were counted after 4 hours of incubation at 37°C. Human macrophages were also tested for ADCC activity using the calcein-AM release assay, but no significant ADCC was measured.

Isolation of cells and phagocytosis was performed as described in detail in (23). In brief, Ficoll-purified normal blood mononuclear cells were incubated in a tissue culture dish for 45 to 90 minutes in monocyte adhesion medium [RPMI 1640 medium supplemented with 7.5% AB serum [Invitrogen], 1% L-glutamine, and 1% penicillin/streptomycin], after which nonadherent cells were removed. After overnight incubation, cells were detached with PBS containing 5 mmol/L EDTA. Detached cells were plated in 8-well chamber slides (LabTek) at  $2 \times 10^5$  cells per well and allowed to differentiate for 7 to 10 days in macrophage culture medium [X-Vivo medium (Lonza), 1% AB serum, 1% L-glutamine, and 1% penicillin/streptomycin]. For measurement of phagocytosis, US7 or TXL2 target cells were added to macrophage at a 1:1 ratio in the presence of 20 µg/mL B-1239 or human IgG as a control. After 2-hour incubation, cells were fixed and stained with Diff Quick (Thermo Scientific) and percentage phagocytosis was determined by counting macrophages having engulfed one or more leukemia cells. At least 200 cells were counted for each experimental condition.

Mouse spleen was mechanically dispersed into a single-cell suspension. After straining through a 70- $\mu$ m strainer, cells were incubated overnight with L929-conditioned RPMI media with 10% FBS, penicillin/streptomycin,  $\beta$ -mercaptoethanol on nontissue culture-treated dishes. Floating cells were removed by washing and cultures were expanded for around 10 days before use. Mouse bone marrow-derived macrophages (BMDM) were obtained using standard procedures. To compare mouse BMDM and spleen-derived macrophages (SDM), human US7 cells were incubated with 25  $\mu$ g/mL B-1239 for 1 hour in complete medium and then washed twice in PBS<sup>-/-</sup>. A total of  $5 \times 10^5$  washed ALL cells were plated into wells containing  $10^5$  macrophages for an E:T ratio of 1:5. Remaining ALL cells were identified after 30 minutes at 37°C using FACS and based on forward scatter (FSC)/side scatter (SSC). TXL2 and TXL2R cells were seeded at  $1 \times 10^6$  cells into 24-well plates containing irradiated  $10^5$  OP9 cells and  $10^5$  SDMs. After addition of 25  $\mu$ g/mL B-1239, cells were incubated for 24 hours. Remaining ALL cells were removed and live cells counted by FACS.

### Human ALL transplant model

All animal experiments were carried out in concordance with Institutional Animal Care and Use Committee and NIH guidelines. Human Ph-positive TXL2 ALL cells were injected at  $2 \times 10^6$  cells per animal into NOD.Cg-Prkdc<sup>scid</sup>Il2rg<sup>tm1Wjl</sup>/SzJ mice (NSG; The Jackson Laboratory). Cells were allowed to proliferate *in vivo* for 6 days before start of treatment. Transplanted mice ( $n = 5$ /group) were either injected i.p. with B-1239  $\alpha$ -BAFF-R antibody (10 mg/kg), orally administered 75 mg/kg of nilotinib, or treated with both antibody and nilotinib for 4 consecutive days. Control mice received peanut butter/oil and control 10 mg/kg human IgG. Mice were sacrificed 12 days after termination of treatment. Bone marrow and spleen were analyzed by FACS for the presence of human ALL cells using human CD19 and CD10 antibodies.

### Statistical analysis

The Student *t* test was performed to assess statistical significance of the *in vitro* results. A *P* value of <0.05 was considered significant. All experiments were done using triplicate wells for each experimental point.

### Results and Discussion

We previously reported that the addition of recombinant BAFF to ALL culture medium protects these cells from chemotherapy-induced apoptosis (1). To further investigate the survival advantage mediated by the BAFF-R to pre-B ALL cells, we generated pre-B ALL cells from bone marrows of WT and *bafl-r* null mutant (KO) mice with a retroviral vector carrying the *BCR/ABL* oncogene. Bcr/Abl expression was confirmed using

Western blot analysis (Fig. 1A). The proliferation and viability of WT and KO pre-B ALL cells were comparable (not shown). Interestingly, WT pre-B ALL cells expressed high levels of the BAFF-R (Fig. 1B), confirming the aberrant appearance of this receptor on ALL cells, as reported by us and others in human ALL (1, 9, 10). We then treated both WT- and KO-deficient leukemic cells with 16 nmol/L nilotinib for 12 days, in the absence of stroma. As shown in Fig. 1C, WT leukemic cells developed resistance to nilotinib in 9 to 10 days, whereas BAFF-R-deficient cells were eradicated. These results establish that the presence of the BAFF-R on pre-B ALL cells confers a survival advantage during drug treatment.

To explore potential therapeutic applications, we inhibited the BAFF/BAFF-R interaction on human ALL cells using either a neutralizing antibody to the BAFF-R (R&D Systems) or a BCMA-Fc decoy receptor, combined with drug treatment. Antibody or BCMA-Fc monotreatment had little effect on cell viability (Fig. 1D and E, left) or proliferation (not shown), but there was a reduction in the viability of ALL cells in the combination treatment group compared with the nilotinib monotreatment group (Fig. 1D and E).

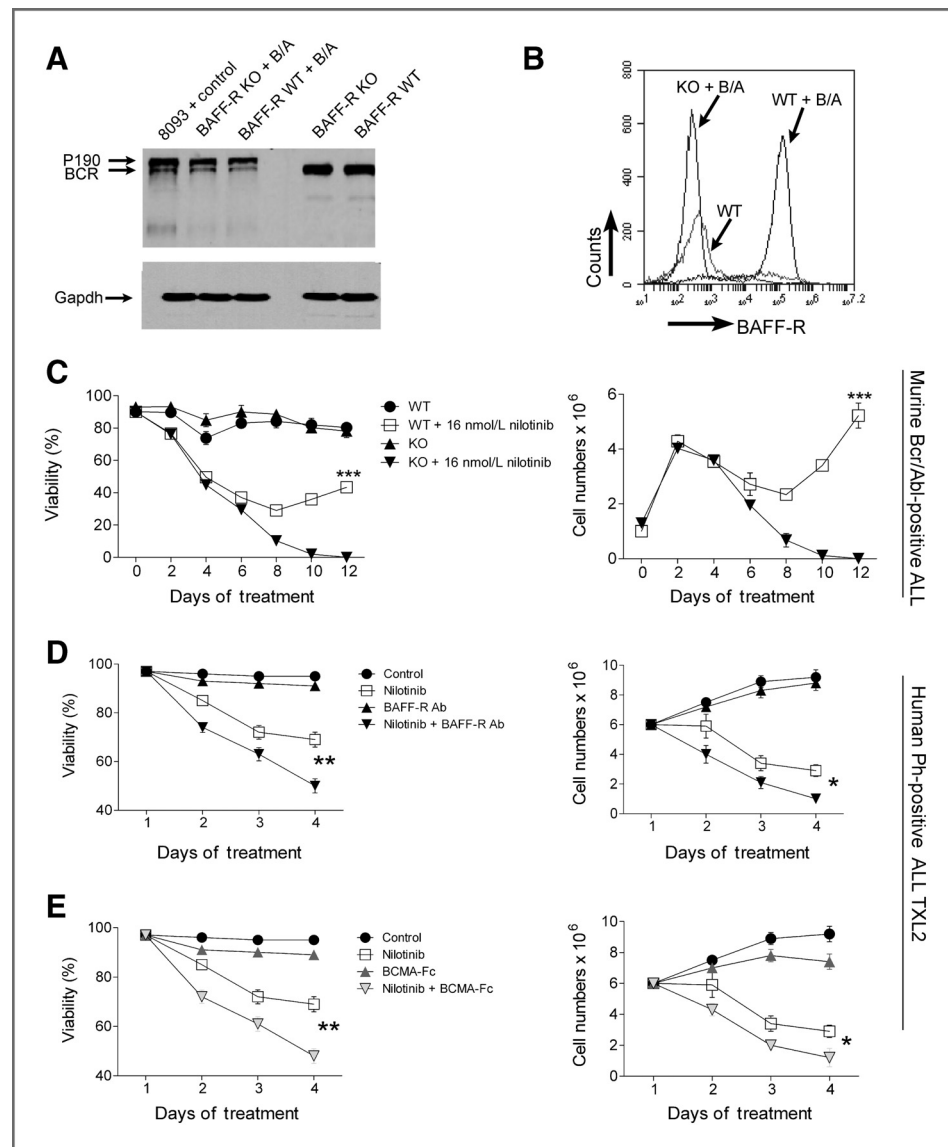
We next characterized B-1239, a human codon-optimized anti-BAFF-R mAb, for possible clinical application based on the presence of the BAFF-R on human pre-B ALL cells. Figure 2A shows that the antibody bound to the BAFF-R on both Ph-positive and Ph-negative ALL cells, as detected indirectly with FITC human IgG. We also compared relative copy numbers of the BAFF-R on a mature B-lineage lymphoma cell line with that on a pre-B ALL. As shown in Fig. 2B, BAFF-R copy numbers present on TXL2 pre-B ALL cells, as measured by the B-1239 antibodies, were around 50% of that of a highly BAFF-R-expressing diffuse large B-cell lymphoma cell line SUDHL4. MFI of BAFF-R expression was also high on normal residual mature lymphocytes present in samples of patients with ALL compared with blasts (average MFI normal BM/PB vs. ALL BM/PB, 4633/5097 vs. 2831/2981, respectively; see Fig. 2C).

The B-1239 antibody inhibited binding of a different anti-BAFF-R antibody in a dose-dependent manner (Fig. 2D). Preincubation of ALL cells with human recombinant BAFF inhibited binding of the B-1239 antibody in a dose-dependent manner (Fig. 2E). These data suggest that the B-1239 antibody binds to epitopes on the BAFF-R that are part of the ligand-binding site.

To address how long the B-1239 antibody remains on the cell surface, we performed a time course to determine when loss of inhibition of binding of the other anti-BAFF-R antibody happens. Figure 2F shows that after 30 hours inhibition is lost. To determine if loss of B-1239 antibody is due to internalization, we imaged cells incubated with Alexa-647-labeled B-1239 using confocal microscopy (Fig. 2G) and ImageStream (Fig. 2H). Both analysis yielded similar results with no detection of internalization of the antibody.



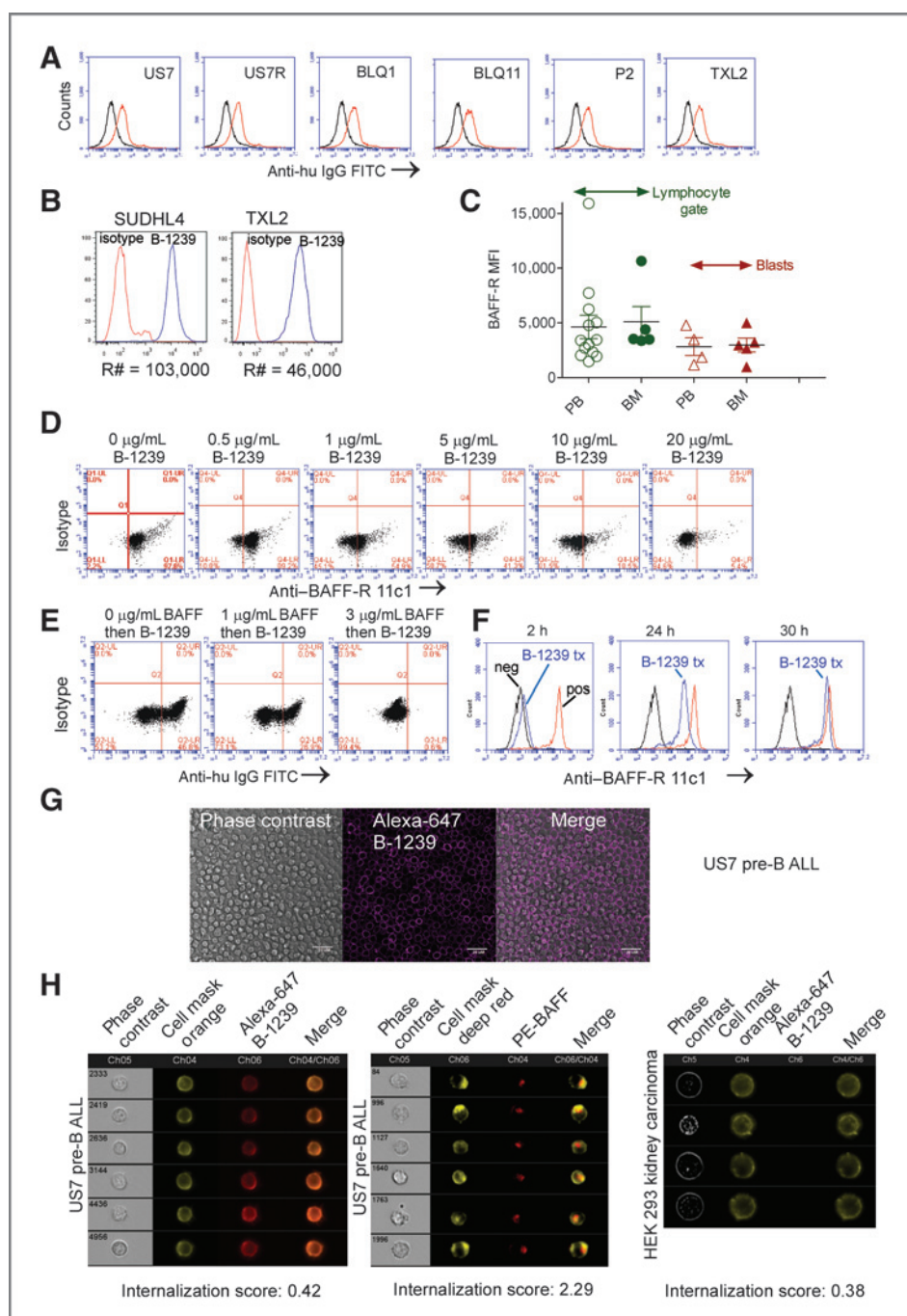
**Figure 1.** Pre-B ALL cells are more vulnerable to therapeutic drug treatment when BAFF-R function is attenuated. **A**, Western blot analysis for Bcr/Abl on WT and BAFF-R-deficient (KO) pre-B cells before and after Bcr/Abl transduction using Bcr N-20 antibodies. Positive control, murine transgenic *BCR/ABL* 8093 ALL cells. **B**, FACS analysis of WT and BAFF-R-deficient pre-B cells before and after Bcr/Abl transduction, using a BAFF-R antibody. **C**, percentage viability (left) and viable cell counts (right) of WT- and BAFF-R-deficient (KO) Bcr/Abl-transduced pre-B cells treated with 16 nmol/L nilotinib for 12 days. \*\*\*,  $P < 0.001$  for nilotinib-treated WT and KO ALL cells. Fourteen independent experiments; all experiments with triplicate samples. **D** and **E**, percentage viability (left) and viable cell counts (right) of human Ph-positive TXL2 ALL cells cocultured with OP9 stroma and treated with function-blocking BAFF-R antibody (5  $\mu$ g/mL; R&D), nilotinib (300 nmol/L), or a combination of both (D) or BCMA-Fc (10  $\mu$ g/mL), nilotinib (300 nmol/L), or a combination of both (E) for 4 days. Control, DMSO vehicle. \*,  $P < 0.05$  and \*\*,  $P < 0.01$  nilotinib versus combination treatment with nilotinib.



Because B-1239 competes with BAFF for BAFF-R binding, we tested if blocking of the BAFF-R with it would enhance chemotherapy sensitivity in these cells. As shown in Fig. 3A and B, this antibody alone also had little effect on cell viability or proliferation. However, there was a reduction in the viability of ALL cells in the combination treatment group, especially in combination with vincristine (Fig. 3B). To address the possibility that nilotinib as tyrosine kinase inhibitor could affect BAFF-R expression, we compared its expression on cells treated for varying periods of time with nilotinib. However, no changes in expression were seen (Fig. 3C).

The cell surface expression of the BAFF-R on ALL cells can be used as a therapeutic target independent of its function: binding of monoclonal antibodies can stimulate cell killing by effector cells that express

the Fc $\gamma$ R (24). Because the B-1239 antibody was designed to promote ADCC, we tested this using NK cells. After comparing different ratios of effector (NK) to target (ALL) cells (not shown), we chose a ratio of 5:1 for ADCC experiments. As shown in Fig. 4A, compared with control human IgG, B-1239 had a dose-dependent specific effect on stimulating NK cell-mediated cytotoxicity. We also tested the ADCC-promoting activity of the antibody on NK cells isolated from different normal donors. Figure 4B shows that depending on the donor NK cells, 20% to 70% of the ALL cells were killed within 4 hours of incubation. Both Ph-positive (BLQ1, BLQ11, P2, and TXL2) and -negative (US7) ALL cells were killed by NK cells. The results shown here validate this novel BAFF-R antibody as a very promising tool for therapy of pre-B ALL.



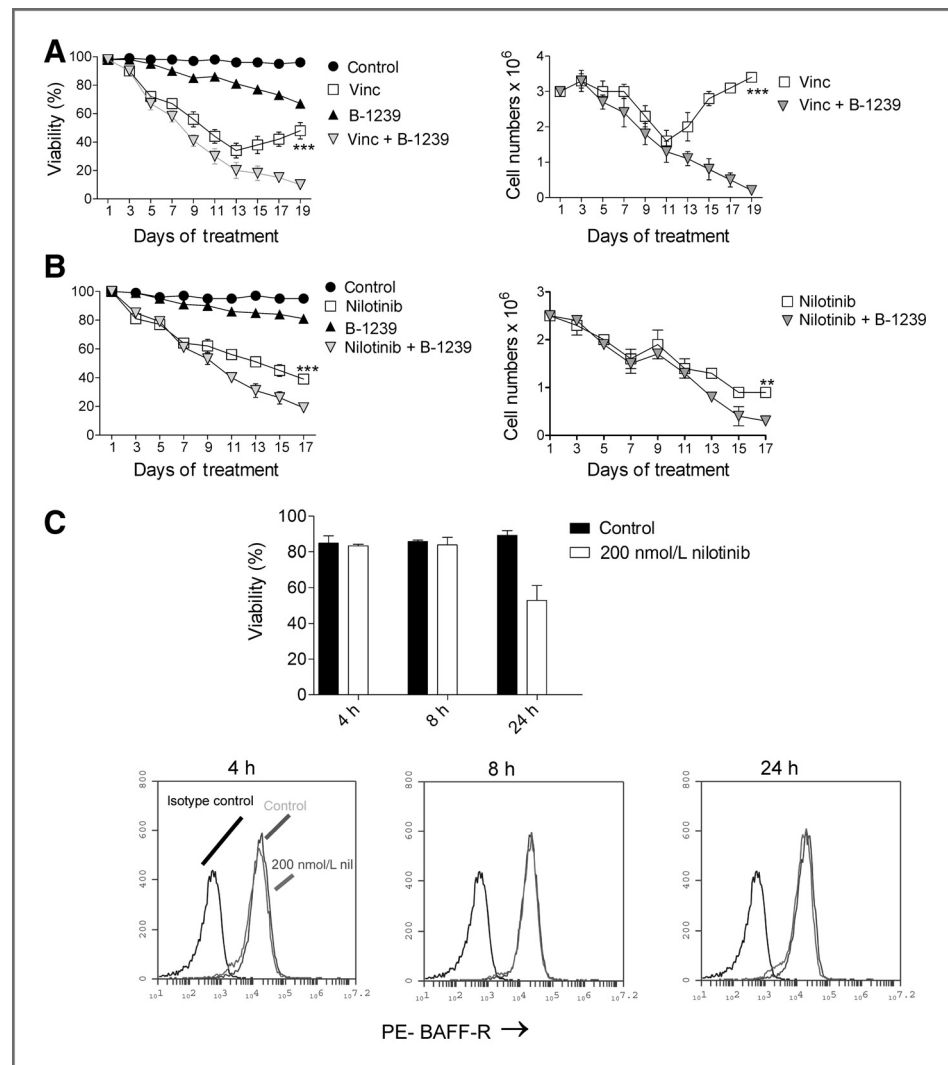
**Figure 2.** Binding of humanized mAb B-1239 to the BAFF-R. **A**, antibody binding of B-1239 (10 µg/mL, 2 hours)  $\alpha$ -BAFF-R antibody to the indicated patient-derived ALL cells detected using FITC anti-human IgG. Black histograms, control anti-human IgG; red histograms, cells first incubated with B-1239, then stained with anti-human IgG antibody. **B**, comparative analysis for BAFF-R copy number (R#) on the surface of SUDHL4 (diffuse large B-cell lymphoma) and Ph-positive ALL TXL2 as measured by B-1239. **C**, comparison of MFI for BAFF-R expression on CD19<sup>+</sup>, BAFF-R<sup>+</sup> residual normal B-lineage cells and CD19<sup>+</sup>, BAFF-R<sup>+</sup> blasts for 4 to 9 different patient samples. Normal non-B lymphocytes did not express the BAFF-R. **D**, binding of PE-labeled anti-BAFF-R antibody 11c1 clone (BD Biosciences) to human US7 ALL cells after preincubation with the indicated amounts of B-1239. **E**, ability of recombinant BAFF to inhibit binding of 5 µg/mL B-1239 to the BAFF-R on US7 ALL cells as detected with FITC anti-human IgG antibody. **F**, cell surface detection as measured by competition of PE-labeled anti-BAFF-R 11c1 antibodies with unlabeled B-1239 mAb on US7 pre-B ALL cells after addition of 30 µg/mL antibody on t = 0. Control, unstained cells; blue, cells treated with B-1239. Red, no B-1239 added. **G**, localization of Alexa-647-conjugated B-1239 antibodies using confocal microscopy. Bar, 20 µm. **H**, representative Amnis ImageStream images showing specific cell surface attachment and lack of internalization of Alexa-627 B-1239 to US7 pre-B ALL cells, compared with positive control internalization of PE-anti-BAFF antibodies, and negative binding control for Alexa-647 B-1239 to HEK 293 cells that lack BAFF-R expression.

Some monoclonal antibodies promote ADCP and macrophages can be a major effector cell type that mediates cell killing through antibodies. We therefore tested human macrophage-mediated cell eradication against TXL2 and US7, representative of Ph-positive and -negative ALLs. As negative controls, we included ICN13, a pro-B ALL, and an AML. On the basis of FACS, neither expresses the BAFF-R (not shown). As shown in Fig. 5A, cell counts of ICN13 and AML incubated with macrophages with and without B-1239

were identical, indicating that in the absence of the BAFF-R these antibodies do not stimulate phagocytosis. However, B-1239 clearly stimulated eradication of ALL cells expressing the BAFF-R, demonstrating the specificity of this effect. We also directly measured phagocytosis and found specific stimulation by B-1239 (Fig. 5B).

As reviewed by Galluzzi and colleagues (14), some targeted drugs may also indirectly decrease tumor cell survival *in vivo* through mechanisms involving immune

**Figure 3.** Combination treatment of pre-B ALL cells with chemotherapy and B-1239 antibodies. **A**, viability (left) and cell counts (right) of US7 ALL cells treated with 20  $\mu\text{g/mL}$  B-1239 (daily), 2.5 nmol/L vincristine (on alternate days), or both over a period of 19 days. **B**, viability (left) and cell counts (right) of TXL2 ALL cells treated with 20  $\mu\text{g/mL}$  B-1239 (daily), 300 nmol/L nilotinib (fresh on alternate days), or both over a period of 17 days. Control, 20  $\mu\text{g/mL}$  human IgG. \*\*,  $P < 0.01$  and \*\*\*,  $P < 0.001$  for vincristine or nilotinib monotreatment compared with combination treatment with B-1239. One of two experiments on triplicate samples. **C**, evaluation of effect of nilotinib treatment on BAFF-R cell surface expression on TXL2 cells. Control, cells treated with DMSO.

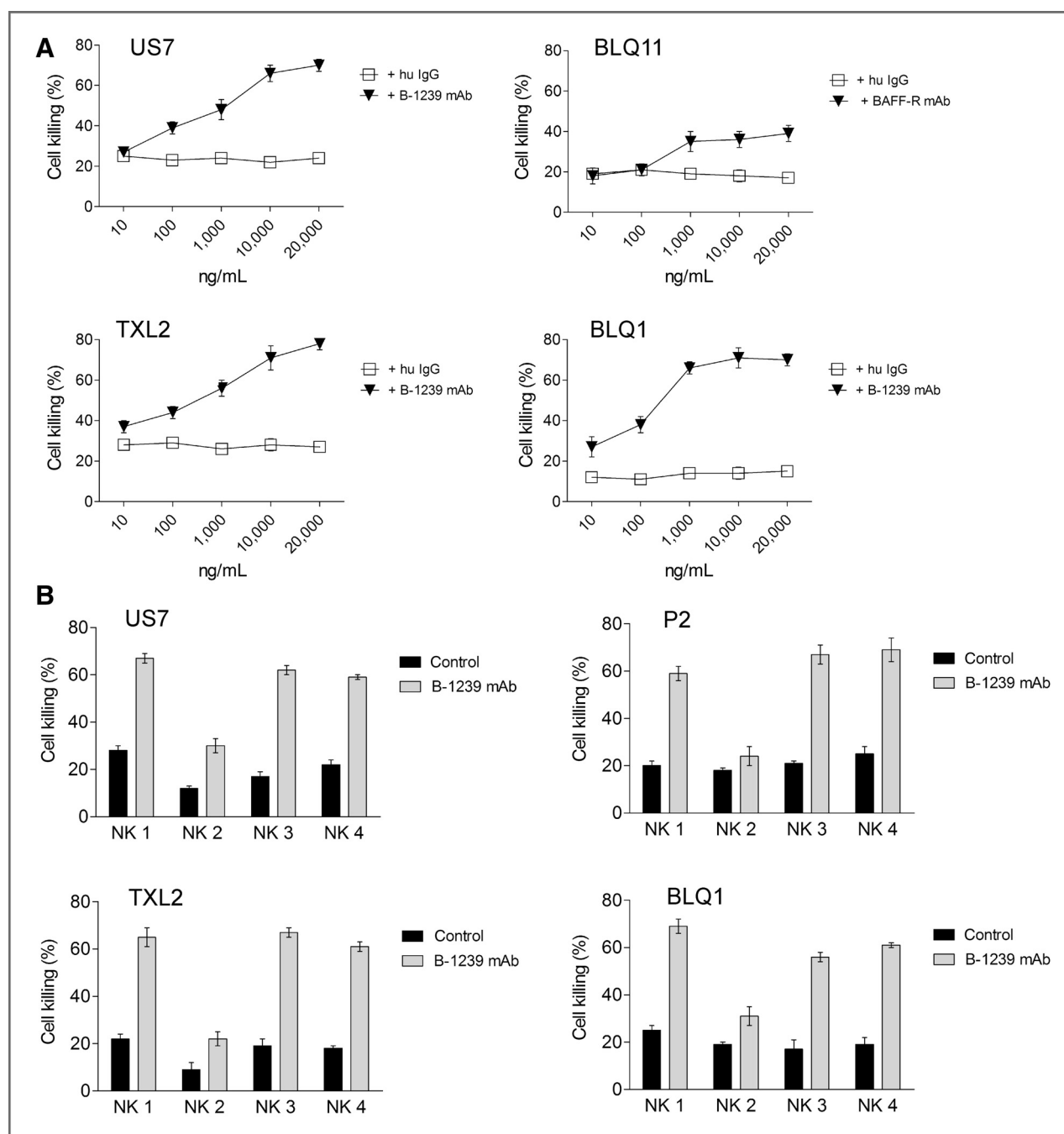


cell function. There is little information if inhibitors such as nilotinib will affect functioning of nonmalignant immune cells. Therefore, we evaluated B-1239-stimulated eradication of TXL2 cells by mouse SDM in the presence of nilotinib. As control, we included TXL2R, which is not responsive to nilotinib. Figure 5C shows that nilotinib treatment did not affect TXL2R cell numbers when there were macrophages present (compare control with nilotinib monotreatment). As expected, B-1239 stimulated phagocytosis of TXL2R, and macrophage ADCP function was not affected by the presence of nilotinib. In contrast and as expected, nilotinib significantly reduced TXL2 cell numbers within 24 hours. B-1239 treatment had a similar effect, and a combined treatment with nilotinib and B-1239 was additive. These results further suggest that nilotinib acts as a chemosensitizer for phagocytosis. Additional experiments with three other Ph-positive pre-B ALLs from relapsed patients, which are nilotinib-insensitive, confirmed that nilotinib acts as a sensitizer, because it

had no additive effect combined with the anti-BAFF-R antibody on these pre-B ALL cells (Fig. 5D).

Major sites of ALL cell infiltration and proliferation include the bone marrow and the spleen. We therefore compared the ability of mouse BMDM and SDM to phagocytose human ALL cells. Figure 5E shows that macrophages from both locations had significant ADCP activity in the presence of B-1239.

To evaluate the ability of B-1239 to stimulate pre-B ALL killing *in vivo*, we transplanted NSG mice with TXL2 cells and allowed the cells to proliferate for 6 days to form an appreciable tumor burden (Fig. 6). Next, either 10 mg/kg B-1239 or human IgG was administered as four daily treatments. We also included treatment groups with 75 mg/kg nilotinib or a combination of nilotinib and B-1239, and all mice were sacrificed to evaluate leukemia cell burden. As shown in Fig. 6, FACS analysis showed that 12 days after the last treatment, leukemia cell numbers in the circulation of control and B-1239-treated groups were comparable. However, a

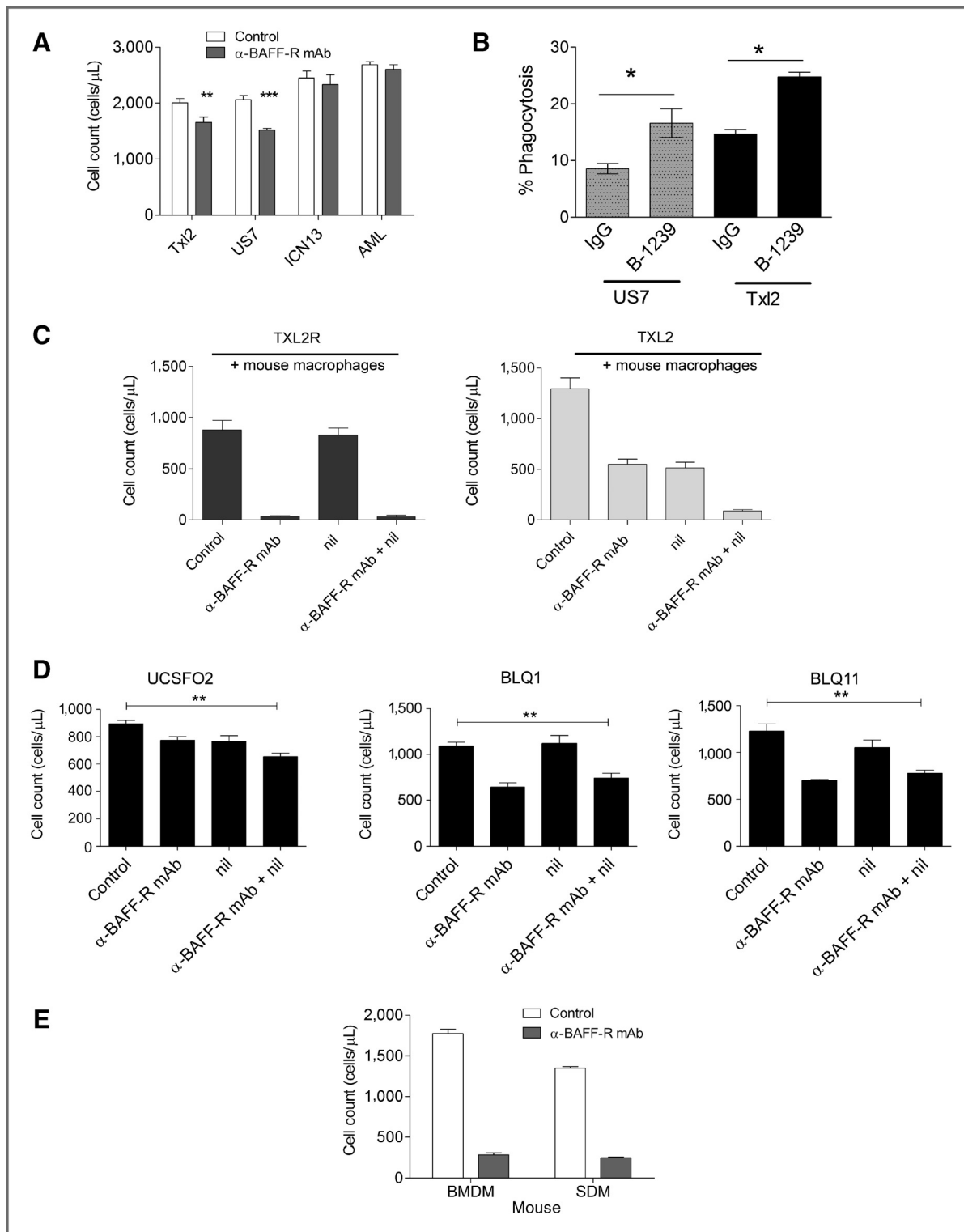


**Figure 4.** B-1239 promotes NK cell-mediated ADCC activity against human pre-B ALL cells. **A**, percentage lysis of ALL cells preincubated with different concentrations of B-1239, after 4 hours of incubation with NK cells. All graphs were generated using an E:T ratio of 5:1 and NK cells from one donor. **B**, ADCC mediated by NK cells from different normal donors (NK 1–NK 4) using an E:T ratio of 5:1 in all graphs and 10  $\mu$ g/mL B-1239. Bars, mean  $\pm$  SEM of triplicate samples.

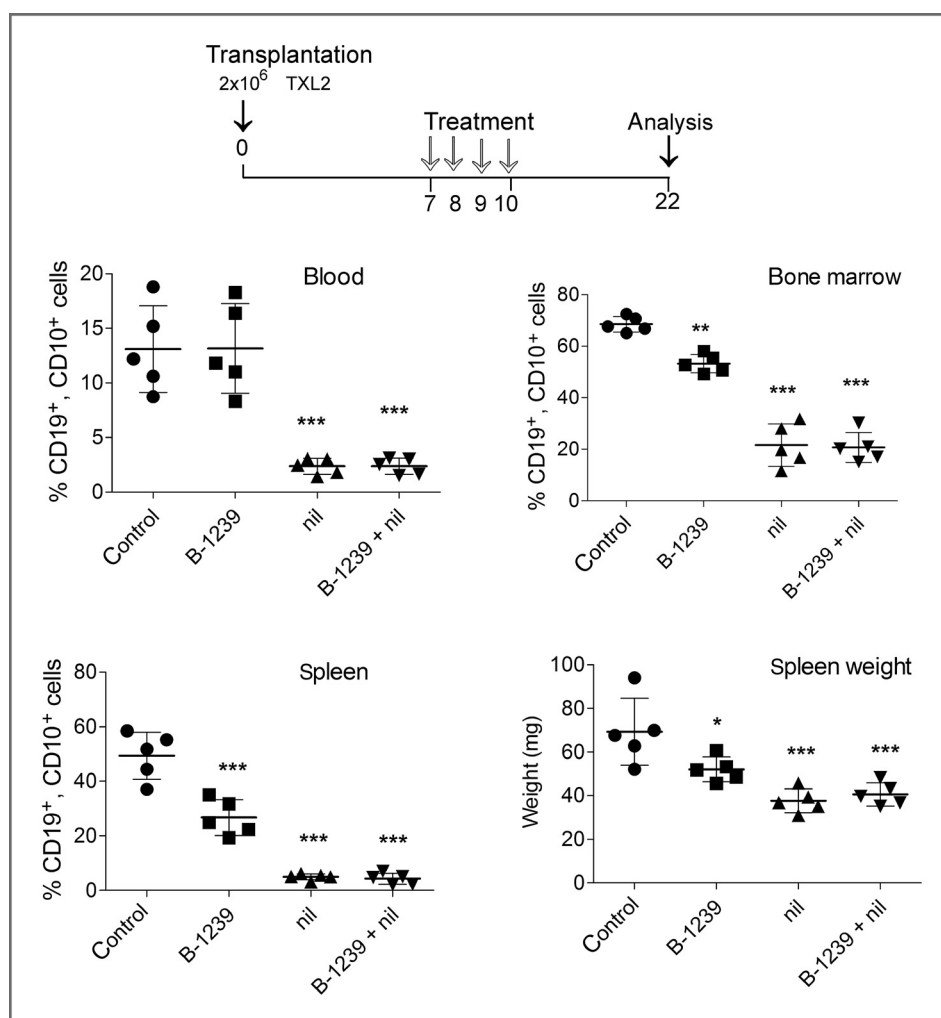
significant inhibition of ALL cell growth in the bone marrow and spleens of mice treated with BAFF-R antibody compared with control groups was noted. The spleen weight of BAFF-R antibody-treated mice was also significantly reduced compared with controls (Fig. 6). Nilotinib monotherapy significantly reduced the ALL cell burden in PB, spleen, and bone marrow.

We previously reported common expression of the BAFF-R in patient-derived B-precursor ALL samples (11), in agreement with Maia and colleagues who detected expression using RT-PCR (9). Because 13 other primary CD19<sup>+</sup>, CD10<sup>bright</sup>, sIgM B-precursor ALL samples were BAFF-R-positive (manuscript in preparation), this receptor may be expressed in a major percentage of





**Figure 5.** B-1239 stimulates ADCP by human and mouse macrophages. **A**, cell counts of TXL2, US7, ICN13, or AML cells preincubated or not (control) with 25 μg/mL B-1239 followed by a 4-hour coinubation with human macrophages. \*\*,  $P < 0.01$  and \*\*\*,  $P < 0.001$  compared with control. **B**, percentage phagocytosis by human macrophages of US7 or TXL2 cells in an E:T ratio of 1:1 after 2 hours in the presence of 20 μg/mL B-1239 or 20 μg/mL control human IgG. One of two experiments with independent macrophage isolates. **C**, live cell counts of TXL2 and TXL2R in the presence of mouse SDM without further treatment (control) or with 100 nmol/L nilotinib, 25 μg/mL B-1239, or both for 24 hours. **D**, live cell counts of the indicated Ph-positive pre-B ALLs treated as in **C**. \*\*,  $P < 0.01$  for combination treatment compared with control. **E**, live cell counts of US7 cells incubated with mouse BMDM or SDM in the presence or absence of 25 μg/mL B-1239.



**Figure 6.** Treatment of mice with B-1239. Top, schematic of *in vivo* treatment with 10 mg/kg B-1239, control 10 mg/kg human IgG, or combined with 75 mg/kg nilotinib. Bottom, percentage of human CD19<sup>+</sup> CD10<sup>+</sup> ALL cells as detected by FACS in blood, bone marrow, and spleen of NSG leukemic mice and spleen weights of control and treated mice on d22. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; all comparisons with control. Nil versus nil+ B-1239, not statistically significant. One of two independently performed experiments.

ALL samples. We conclude that B-1239, which interferes with binding of BAFF to the BAFF-R, is a promising antibody-based treatment that should be further evaluated because it may reduce the fitness of pre-B ALL cells when combined with other drugs. Importantly, it has very significant other activities, including ADCP *in vitro* and *in vivo*, and promotes NK cell-mediated killing of ALL cells. Together, these activities are likely to be extremely useful to eradicate pre-B ALL cells.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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